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Trace Element Metabolism in Children with Menkes' Syndrome

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Summary

Menkes' syndrome, or the kinky hair syndrome, is a hereditary, progressive disease caused by an X-linked recessive gene. The basic defect has been attributed to an insufficient intestinal absorption of copper.

Observation of typical signs of Menkes' syndrome in neonates, however, indicates the possible presence of a prenatal defect in the metabolism of copper.

Very little reliable information is available on the distribution of copper and other trace elements in foetuses of different age, and the sampling of tissue from a foetus suspected of Menkes' disease was therefore supplemented by sampling a number of controls of different gestational age.

The analysis of samples from a total of 7 foetuses of 15-21 weeks' gestational age was carried out by neutron activation analysis with radiochemical separation, so that simultaneous determination of Cu, As, Se, and Mn was achieved.

The analytical procedure was investigated by the Analysis of Precision, and its performance characteristics were ascertained. Accuracy was checked by the analysis of Standard Reference Materials.

Results for copper in µg per g wet weight are summarized in the accompanying table.

	Menkes' foetus	Range of controls	No. of controls
Kidney	17.3	0.5 - 1.1	4
Spleen	15.4	0.7 - 1.3	4
Pancreas	15.3	1.3 - 1.7	3
Placenta	14.5	0.8 - 2.2	3
Brain	1.0	0.3 - 0.5	4
Lung	1.8	0.3 - 0.6	4
Muscle and skin	2.6	0.4 - 1.0	4
Liver	11.8	33.2 -37.9	4

As described in a paper published under the contract in question, the distribution of copper among the organs analyzed from the foetus expected to develop Menkes' syndrome is entirely different from the distribution observed in the corresponding controls. In particular, the concentration in the liver was much lower, whereas all other tissues had concentrations above normal.

Similar differences were not found for the concentrations of As, Se, and Mn in the foetuses investigated, and the distribution of these elements was not very different from that in adults.

These observations do not support the hypothesis of defective intestinal transport of copper as the primary cause of Menkes' syndrome, nor do they indicate an inadequate placental transport of copper to the foetus. Clearly, a search must be made for a metabolic defect that also affects the intrafoetal transport.

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INTRODUCTION

The discovery by Danks et al. (1972) of a connection between low copper concentrations in plasma and the genetic defect characterized as Menkes' syndrome, led to a sudden interest in reliable determinations of copper in biological samples.

It was therefore decided to extend our current method for the determination of As, Se, and Mn by neutron activation analysis (Heydorn 1972) to include the determination of Cu, so that all 4 trace elements are determined in the same sample.

In order to minimize the effort connected with a thorough documentation and investigation of the performance characteristics of an analytical method, the previous procedure for As, Se, and Mn should remain virtually unchanged and thus its characteristics be directly applicable to the revised method for 3 out of the 4 elements.

The starting point of the extension is therefore the copper containing precipitate of iodides and cupferrates, from which the other 3 elements have been separated. According to the separation scheme (Heydorn 1972) this precipitate contains a dozen elements, but only a few have high effective values, in particular tungsten and antimony.

Separation of copper from these elements is achieved by precipitation with thioacetamide in ammoniacal solution (Finston 1955).

Incorporation of this procedure into our previous method did not increase the time needed for separation.

ANALYTICAL METHOD

Our previous method for the determination of arsenic, manganese and selenium in biological materials calls for a one-hour irradiation of a one-gram sample followed by radiochemical separation and measurement of the activities of ^{76}As, ^{56}Mn and ^{81m}Se in scintillation detectors. The chemical yield is determined by added ^{54}Mn, respectively re-irradiation of the separated arsenic and selenium samples.

In addition, copper is now determined using ^{64}Cu as indicator, and the chemical yield by re-irradiation of the separated copper samples.

Determination of Arsenic, Manganese and Selenium

No changes are required in the analytical procedure for the three original elements. However, simultaneous irradiation of a bromine reference together with the 4 comparator standards was found useful when correcting for ^{82}Br at very low arsenic concentrations (Damsgaard 1973).

Determination of Copper

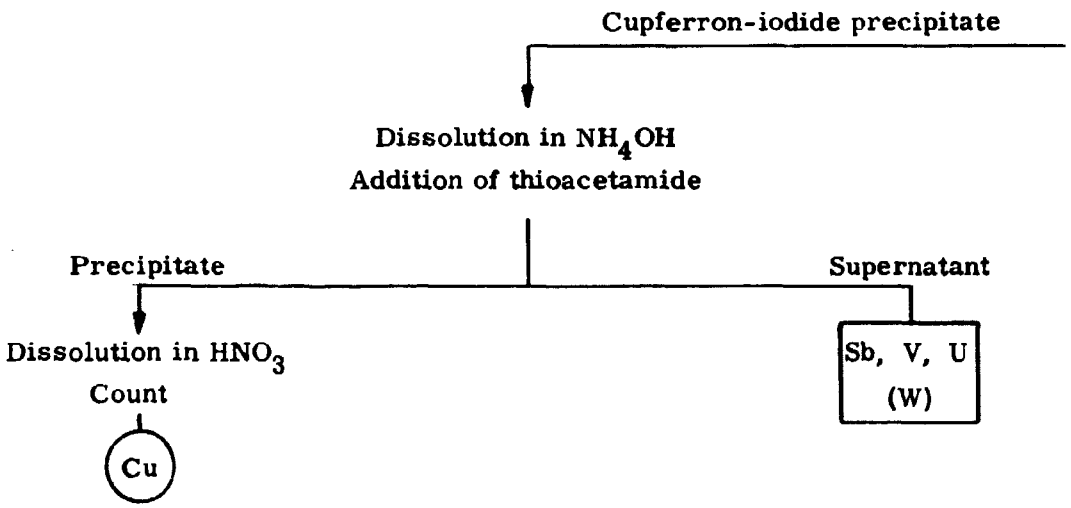
Copper is removed from the other elements as cuprous iodide in the precipitate resulting from the addition of potassium iodide and cupferron.

Separation of copper from antimony, as well as from traces of ^{24}Na, is achieved by dissolution of the precipitate in ammonium hydroxide and precipitation of the sulphide with thioacetamide.

Counting of ^{64}Cu is based on the 511 keV annihilation peak in spite of its poor specificity; the only Y-ray of 1345 keV is subject to strong interference from traces of ^{24}Na, unless counted with a Ge(Li) detector (Maziére 1972).

Procedure

The following description is an overlay to our previous procedure so common features are omitted; a schematic presentation is shown below.



Reagents

Ammonium hydroxide 4M
Nitric acid 65%

Comparator Standards

Cu-comparator, 5 µg Cu/ml
prepared by dilution of Cu-carrier with 0.1 N HNO_3

The addition of nitric acid improves the stability, so that the change of concentration is reduced from several percent to well below 1% per month.

Irradiation

The sample is irradiated together with comparator standards of all 4 elements in heat-sealed half-dram polyvials for 1 hour, as shown on the accompanying figure 1.

A ^{82}Br reference is produced by simultaneous irradiation of about 1 µg of Br as NH_4Br in 1 ml aqueous solution.

Decomposition

The sample is decomposed as previously described; 1000 µl of Cu-carrier is transferred to a half-dram polyvial, which is heat-sealed and set aside for yield determination.

Separation of Copper

The precipitate from the addition of potassium iodide and cupferron is washed twice with water and dissolved on the filter* in 20 ml of ammonium hydroxide.

About 100 mg of thioacetamide are added to the filtrate, and copper is precipitated as the sulfide by gentle heating in a water bath.

After centrifugation the supernatant is discarded, and the precipitate is dissolved in 1.0 ml of nitric acid. Finally, the solution is transferred to a half-dram polyvial, which is then heat-sealed and ready for counting.

Counting of Copper-64

A comparator standard is made by transferring 1000 µl of the irradiated Cu-comparator to a half-dram polyvial.

The copper sample is counted for at least 4 minutes live time about 24 hours after pile-out with a 3"x3" scintillation detector at a gain of 6.7 keV/channel. The comparator standard is counted for 4 minutes under the same conditions.

Yield of Copper

The separated copper sample and the Cu-carrier sample set aside for yield determination are irradiated together in the reactor for 10 seconds.

About 24 hours after pile-out each sample is counted for 4 minutes under the same conditions as above.

The chemical yields of copper averaged 70%.

Calculation of Result

Copper is determined from the 511 keV peak areas of sample and comparator corrected for differences in decay and counting times.

The chemical yield is calculated in the same way from the spectra of the re-irradiated sample and carrier.

The copper content, corrected for chemical yield, is converted into nanograms.

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*Munktell 20 H (recommended by Berzelius)

ANALYTICAL EVALUATION

The errors originating from systematic differences in neutron flux density between sample and comparator can be cancelled by re-irradiation yield determination in the same positions. Random errors from the uncertainty of positioning of the irradiation container during activation are unavoidable, but their magnitude should be reduced the closer sample and comparator are.

In the present case copper was chosen as the most favoured element, its comparators occupying the position closest to the sample.

Interference

The analytical procedure for arsenic, manganese and selenium is unchanged, and consequently interference from other elements with the determination of these three elements is the same as previously reported (Heydorn 1972).

The measurement of ^{64}Cu by the 511 keV annihilation peak is particularly susceptible to interference, not only from radionuclides emitting γ-rays with neighbouring energies, but also from all nuclides with γ-transitions greater than 1022 keV producing electron-positron pairs.

Experimentally determined interferences are therefore particularly important in this case, and effective values for 7 elements are presented in table 1.

Blank

The half-dram polyvials release up to a few nanograms of manganese to a water sample during irradiation, which is important in the analysis of serum (Damsgaard 1973). Cleaning with a 3% hydrogen-peroxide solution produced the lowest and most consistent results, but the use of an alternative polyethylene ampoule with a volume of 5 ml reduced the problem to insignificance.

Results of a similar investigation for copper are summarized in table 2 and show that a single rinsing with redistilled water is superior to the classical nitric acid procedure (Robertson 1972) as well as to the hydrogen-peroxide treatment.

Table 1

Experimentally determined interferences in copper analysis

Interfering element	Radioactive tracer	Activity µCi	Mass µg	Separation factor S	Effective value f	ppm of element ~ ± 1 ppb of Cu
Na	Na-24	*		1.4×10^{-5}	0.033	2,000
Zn	Zn-65	12	1000	1.4×10^{-3}	-0.016	40
Ga	Ga-72	200	6	3.7×10^{-1}	-0.13	0.02
As	As-76	25	**	1.3×10^{-3}	0.93	0.8
Br	Br-80m	16	1.2	5.0×10^{-5}	0.32	60
Sb	Sb-122	3	**	2.6×10^{-2}	0.27	0.15
W	W-187	10	0.1	1.5×10^{-3}	0.96	0.7

* Irradiated sample

** Irradiated carrier

Table 2

Copper blank values for different irradiation containers

Irradiation container		Blank value			
Type	Supplier	Total Cu ng	Volume ml	Number of analyses	Concentration of Cu ng/ml
Polyvial		122 ± 52	1.1	4	2.8 ± 0.6
H_2O_2 cleaned	Olympic Plastics Company	132 ± 66		4	11 ± 3
HNO_3 cleaned		101 ± 15		10	33 ± 9
Polyethylene ampoule	Atomic Industrial Co.		4.5	6	7.2 ± 0.9

PRECISION AND ACCURACY

Estimation of Precision

Random variations in neutron fluence between sample and comparators give rise to a standard deviation of 5% for arsenic and selenium, where the chemical yield is determined by re-irradiation.

For manganese the corresponding standard deviation is only 3.5%, the contribution from yield determination by the counting of ^{54}Mn being included in the counting statistics.

For copper the random flux density variation is reduced to insignificance by the favourable irradiation position shown in fig. 1. The a priori precision for Cu is therefore unknown.

Counting statistics are calculated as usual; annihilation of positrons from ^{64}Cu was found to take place within the liquid samples, and no special absorber was needed.

The overall precision of individual results was calculated as the combined effect of counting statistics and flux density variation.

Analysis of Precision

The analysis of Standard Reference Materials permits the simultaneous determination of precision and accuracy, but only 2 such materials are available; additional verification of the calculated precision was therefore provided by the analysis of Mediterranean oyster homogenate, which is distributed by the International Laboratory of Marine Radioactivity as part of an intercalibration exercise.

Results for the analysis of SRM 1571 and SRM 1577 are presented in table 3 along with an Analysis of Precision (Heydorn 1973) based on 25 results for 3 elements. Results for the Intercomparison Sample MA-M-1 are given in table 5 with the Analysis of Precision based on 14 results for all 4 elements.

Results from the analysis of Orchard Leaves agree with the assumption that random variations in neutron fluence between sample and comparators of copper have been reduced to insignificance compared to a 1% relative standard deviation from counting statistics.

Table 3

Analysis of Standard Reference Materials

Material analysed	Number of samples	Element determined	A priori precision	T	d.f.	Mean value ppm	Certified value ppm
Orchard Leaves[a)]	4	Cu	-	1.44	2	11.70 ±0.05	12±1
Bovine Liver[b)]	9	Cu	-	19.85	4	186 ±2	193±10
	6	Mn	3½ %	3.47	3	9.30 ±0.15	10.3±1.0
	6	Se	5%	3.19	3	1.09 ±0.03	1.1±0.1
		Analysis of Precision		27.95	12	$P(\chi^2 > T) = 0.005$	

a) National Bureau of Standards, Reference Material 1571, 1971

b) National Bureau of Standards, Reference Material 1577, 1972

Results from the analysis of SRM 1577 Bovine Liver on the other hand demonstrate that unexpected sources of variation influence results for Cu, but probably not for Mn and Se; their magnitude was estimated at ±3% relative.

If we disregard errors that would affect all 3 elements to the same degree, the only methodological errors envisaged are irreproducible irradiation geometry, and inferior radiochemical separation leading to variable interference from other radionuclides.

The latter possibility was checked by re-counting the separated samples for ^{64}Cu on a Ge(Li) detector, where interference is limited to contributions from high-energy γ-emitters. Results for 7 replicate countings are given in table 4 together with their standard deviation based on counting statistics only.

Table 4

Duplicate countings of ^{64}Cu samples from the analysis of SRM 1577

Sample weight (freeze dried) mg	Counting on NaI(Tl) detector ppm Cu	Ge(Li) detector ppm Cu
230.3	184.3 ± 2.9	189.3 ± 1.6
289.5	176.4 ± 2.6	176.1 ± 1.4
336.2	194.7 ± 2.5	191.8 ± 1.5
301.7	184.1 ± 4.1	188.4 ± 1.5
285.0	178.0 ± 2.6	180.6 ± 1.9
282.3	188.0 ± 2.8	189.9 ± 1.4
289.3	192.1 ± 3.0	186.0 ± 1.3
Number of duplicates	7	
T	8.75	
$P(\chi^2 > T)$	0.27	

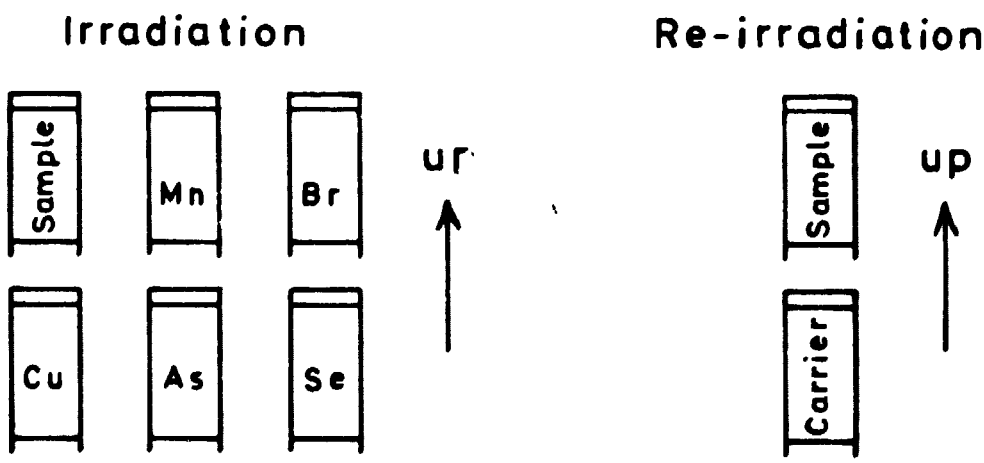
Analysis of Precision of these results does not indicate the presence of sources of variation other than counting statistics; the great difference in counting geometry also affects interference from high-energy γ-emission, so this type of error would also be detected.

Thus, no sign of inferior radiochemical separation could be found by this method. In addition, the results for SRM 1571 make it unlikely that variation in irradiation geometry could be responsible for a ± 3% a priori error.

The most likely source of variation may therefore be beyond our control and stem from lack of homogeneity of the element Cu in the sample material. The certificate of analysis for SRM 1577 estimates the variation between samples weighing at least 250 mg to be within ± 5 percent relative for the trace elements investigated.

No positive identification of the unexpected variation in the results for Cu in Bovine Liver was made, and it is therefore impossible to decide under what circumstances it should be included in the a priori precision for the analysis of biological samples in general.

In such cases the inclusion should be made so that the risk of committing errors of the second kind is not increased; this means that in cases where the homogeneity is better than in SRM 1577 - for example SRM 1571 - we may get significantly low values for T in the Analysis of Precision. Hereby, the sensitivity of the test for detecting other sources of variation is correspondingly reduced.



Positions of sample and comparators during activation

Figure 1

Precision of Results

The Mediterranean oyster homogenate was chosen as Reference Material for the analysis of all 4 elements, and results are presented in table 5.

When re-irradiation of sample and comparator is carried out as shown in figure 1, i.e. one analysis at a time, the random variation in flux density is minimized, and no longer contributes to the a priori precision of arsenic and selenium.

For Cu the unidentified variation of $\pm 3\%$ has to be included in the a priori precision, and consequently all 4 elements may be assigned the same a priori precision of $3\frac{1}{2}\%$.

This convenient assumption has been made for the Analysis of Precision of the 14 results for Sample MA-M-1 in table 5, and no significant disagreement could be found.

Table 5

Analysis of Mediterranean oyster homogenate, Code MA-M-1

Element determined	Number of samples	T	d.f.	Mean value ppm
As	3	0.66	1	12.3 ± 0.3
Cu	4	2.75	2	331 ± 6
Mn	3	0.08	1	68.2 ± 1.5
Se	4	2.11	2	2.26 ± 0.08
Analysis of Precision		5.61	6	$P(\chi^2 \geq T) = 0.47$

Accuracy

Results for Mn and Se in Orchard Leaves were reported by Heydorn (1972), and values for As were the subject of a special report (Damsgaard 1973).

Results for Mn and Se in Bovine Liver are given in table 3 together with the certified values from the National Bureau of Standards; here the same pattern is observed: excellent agreement for Se, and Mn at the lower limit of the confidence interval.

For Cu the agreement is satisfactory for the two materials, although both values are about 3% lower than the certified mean. The unknown source

of variation in the analysis of SRM 1577 does not appear to cause any significant bias of the results, which is in agreement with the assumption that lack of homogeneity is the original cause of the trouble.

Nuclear Interference

For arsenic, manganese and selenium, the interference from nuclear transmutation caused by fast neutrons has been reported previously (Damsgaard 1973). More recent cross sections for (n, p) and (n, α) reactions (Calamand 1974) in Se, Br, and Co reduce the estimated interference to even less importance.

Nuclear interference is independent of irradiation time, and the previous experimentally determined values for Fe and Br are directly applicable to the present method.

The only transmutation reaction producing ^{64}Cu is $^{64}Zn(n, p)^{64}Cu$, and the calculated interference based on a thermal to fast neutron ratio of 44 is 1 ppb of Cu produced by 9 ppm of Zn.

Blank Correction

The blank values for arsenic and selenium are below the detection limits, whereas for manganese and copper this may not be the case.

For polyvials cleaned by nitric acid, there is no doubt that the blank values stem from the irradiation container, but on the other hand the limited contact between a tissue sample and the container wall makes a tissue blank lower than that of water, and results from the analysis of tissue samples in this report were not corrected for container blanks.

RESULTS

The primary purpose of the present investigation was to quantify the difference in Cu concentrations between a foetus suspected of Menkes' disease and normal foetuses of comparable gestational age.

With the exception of the liver, foetal organs are not well investigated for their trace element content, because of the limited sample size and the lack of sensitivity of most classical methods of analysis. No really reliable control values for Cu were found, and values for As were practically non-existent.

It was therefore decided to analyse those foetal organs, for which previous results were available for normal, adult individuals, not only for Cu, but for As, Se, and Mn as well.

Table 6

Concentrations of copper in foetal organs in µg/g wet tissue

Case No.	191	193	16	18	98	126	127
Liver	11.8	37.9	29.5	34.1	17.5	37.0	33.2
Brain	1.04	0.35	0.39	0.52	0.29	0.35	0.27
Lung	1.84	0.51	0.52	0.57	0.49	0.35	0.41
Muscle and skin	2.57	0.45	-	1.02	0.45	0.43	0.99
Spleen	15.4	0.74	0.62	1.15	0.90	0.89	1.27
Kidney	17.3	0.80	0.74	1.14	0.80	0.52	0.84
Pancreas	15.3	1.74	1.33	1.60	1.12	-	1.27
Intestine	-	-	5.50	4.18	6.55	7.48	4.06
Placenta	14.5	0.84	2.24	0.98		0.76	
Amniotic fluid	0.29	0.24					
Cord erythrocytes *	0.62	0.17					
Maternal serum	2.58	1.92					

*Total quantity in µg

Table 7

Concentrations of selenium in foetal organs in µg/g wet tissue

Case No.	191	193	16	18	98	126	127
Liver	0.24	0.30	0.31	0.33	0.20	0.22	0.09
Brain	0.05	0.10	0.08	0.09	0.07	0.08	0.07
Lung	0.13	-	0.10	0.12	0.11	0.09	0.10
Muscle and skin	0.10	-	-	0.09	0.04	0.06	0.07
Spleen	0.23	0.26	0.20	0.08	0.24	0.30	0.16
Kidney	0.12	0.13	0.18	0.19	0.18	0.17	0.12
Pancreas	0.13	0.12	0.18	0.22	0.05	0.09	0.13
Intestine	-	-	0.14	0.12	0.14	0.11	0.18
Placenta	0.17	0.26			0.16	0.15	
Amniotic fluid	n.d.	n.d.					
Cord erythrocytes *	0.02	0.03					
Maternal serum	0.03	0.04					

*Total quantity in µg

Table 8

Concentrations of manganese in foetal organs in ng/g wet tissue

Case No.	191	193	16	18	98	126	127
Liver	712	651	437	559	428	635	876
Brain	105	115	111	123	108	142	121
Lung	87	80	86	77	117	85	80
Muscle and skin	45	72	-	21	78	55	173
Spleen	94	133	137	119	87	155	151
Kidney	195	179	215	195	260	211	179
Pancreas	461	399	308	165	219	507	330
Intestine	-	-	539	945	1114	1184	945
Placenta	58	65	34	54		78	
Amniotic fluid	5.5	7.1					
Cord erythrocytes*	5.4	9.8					
Maternal serum	1.8	1.8					

*Total quantity in ng

Table 9

Concentrations of arsenic in foetal organs in ng/g wet tissue

Case No.	191	193	16	18	98	126	127
Liver	1.33	9.7	1.72	2.1	3.4	0.49	5.8
Brain	0.50	1.14	1.35	0.64	2.0	0.50	1.9
Lung	0.56	2.2	1.8	1.5	2.4	0.3	2.0
Muscle and skin	6.3	3.7	-	1.67	3.1	0.41	3.6
Spleen	n.d.	2.8	2.1	0.9	5.7	1	3.6
Kidney	1.1	3.1	1.6	1.6	2.9	1.3	2.6
Pancreas	1.7	2.7	1.4	1.3	9.6	1	5.4
Intestine	-	-	1.25	1.29	7.0	0.69	3.3
Placenta	1.5	2.9	1.2	0.2		1.1	
Amniotic fluid	35.7	45.7					
Cord erythrocytes*	0.5	0.6					
Maternal serum	0.7	1.5					

*Total quantity in ng

Table 10

Data for foetuses included in the investigation

Case No.	191	193	16	18	98	126	127
Sex	M	F	M	M	M	M	M
mm CR length	145	175	155	200	130	98	130
mm CH length	212	260	225	280	185	143	185
Weight	200 g	360 g	247 g	448 g	112 g	74 g	128 g
Abortion by	sectio parva	sectio parva	prostaglandine	not known	spontaneous	sectio parva	sectio parva
Indication	Menkes' syndrome	12 trisomi	not known	mental		mental	social

The scarcity of abortions performed in normal pregnancies after 3 months' gestational age made it reasonable to include in the investigation samples from foetuses that were not strictly applicable as controls.

Results are presented in the preceding tables together with other pertinent information for judging the value of the data.

CONCLUSION

The distribution of copper among the organs of the foetus expected to develop Menkes' syndrome was found to differ significantly from the distribution in other, normal foetuses, aborted in the same way (Heydorn et al. 1975), and of comparable gestational ages.

The elements selenium, manganese, and arsenic did not show corresponding changes in their distribution, nor did they display any striking differences from the results for normal adults reported by Larsen et al. (1972).

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